Annals of Botany **98**: 157–163, 2006
doi:10.1093/aob/mcl081, available online at www.aob.oxfordjournals.org

Expressed Sequence Tags (ESTs) and Phylogenetic Analysis of Floral Genes from a Paleoherb Species, *Asarum caudigerum*

YINHE ZHAO[1,2,3], GUOYING WANG[2], JINPENG ZHANG[2], JUNBO YANG[1], SHANG PENG[1], LIANMING GAO[1], CHENGYUN LI[3], JINYONG HU[4], DEZHU LI[1,*] and LIZHI GAO[5]

[1]*Laboratory of Biodiversity and Plant Biogeography, Kunming Institute of Botany, Chinese Academy of Sciences, Kunming 650204, China,* [2]*State Key Laboratory of Agrobiotechnology, China Agricultural University, Beijing 10-Institute for Plant Breeding Research, Carl-von-Linn0094, China,* [3]*College of Agronomy and Key Laboratory for Plant Pathology of Yunnan Province, Yunnan Agricultural University, Kunming 650201, China,* [4]*Max-Plancke Weg 10, Koeln 50829, Germany and* [5]*Department of Biology and Biochemistry, University of Houston, Houston, TX 77204, USA*

Received: 31 October 2005 Returned for revision: 30 January 2006 Accepted: 9 March 2006 Published electronically: 4 May 2006

• *Background and Aims Asarum caudigerum* (Aristolochiaceae) is an important species of paleoherb in relation to understanding the origin and evolution of angiosperm flowers, due to its basal position in the angiosperms. The aim of this study was to isolate floral-related genes from *A. caudigerum*, and to infer evolutionary relationships among florally expression-related genes, to further illustrate the origin and diversification of flowers in angiosperms.
• *Methods* A subtracted floral cDNA library was constructed from floral buds using suppression subtractive hybridization (SSH). The cDNA of floral buds and leaves at the seedling stage were used as a tester and a driver, respectively. To further identify the function of putative MADS-box transcription factors, phylogenetic trees were reconstructed in order to infer evolutionary relationships within the MADS-box gene family.
• *Key Results* In the forward-subtracted floral cDNA library, 1920 clones were randomly sequenced, from which 567 unique expressed sequence tags (ESTs) were obtained. Among them, 127 genes failed to show significant similarity to any published sequences in GenBank and thus are putatively novel genes.
• *Conclusions* Phylogenetic analysis indicated that a total of 29 MADS-box transcription factors were members of the *APETALA3(AP3)* subfamily, while nine others were putative MADS-box transcription factors that formed a cluster with MADS-box genes isolated from *Amborella,* the basal-most angiosperm, and those from the gymnosperms. This suggests that the origin of *A. caudigerum* is intermediate between the angiosperms and gymnosperms.

Key words: Expressed sequence tags (ESTs), *Asarum caudigerum*, floral genes, suppression subtractive hybridization (SSH), MADS-box gene, phylogenetic reconstruction.

INTRODUCTION

The flower is the most important organ in the growth and development of plants. From an evolutionary standpoint, it has long been a predominant source of characters for angiosperm taxonomy. In over 250 000 angiosperm species on Earth today, the flowers come in a wide variety of sizes, shapes and colours (Thorne, 1992; Irish, 2003). Despite the diversity of their forms, flowers in general have a very simple and regular organization. Flowers arise from a florally determined meristem, whose cells proliferate to form the floral organs. Typical angiosperm floral organs consist of sepals, followed by petals, stamens and, last, carpels, each of which have been interpreted as modified leaves (Meyerowitz *et al.*, 1989; Pelaz *et al.*, 2001; Ditta *et al.*, 2004). These floral organs are developed from homeotic genes, and they are expressed in floral organ primordia. Many of these homeotic genes are from the MADS-box gene family, which encode transcription factors (Weigel, 1998; Theissen *et al.*, 2000; Theissen, 2001; Becker and Theissen, 2003).

It has been recognized that the parts of floral organs in a typical eudicot flower are regulated by A, B and C class genes; thus the ABC model was proposed according to this theory (Bowman *et al.*, 1991; Coen and Meyerowitz, 1991). The model indicates that floral organ identity is established by overlapping functions of three classes of gene activity: A alone determines sepal identity; A and B together determine petal identity; B and C together determine stamen identity; and C alone determines carpel identity (Coen and Meyerowitz, 1991). Class D genes, specifying ovules, were later added to the ABC model (Ferrario *et al.*, 2003). However, the ABC genes are not sufficient for floral organ identity since ectopic expression of these genes fails to convert vegetative leaves into flower organs (Mizukami and Ma, 1992; Krizek and Meyerowitz, 1996). This finding suggests that normal floral organ development also requires another class of floral hemeotic genes, termed class E genes (SEPALLATA1/2/3/4, or SEPs; Pelaz *et al.*, 2000, 2001; Ditta *et al.*, 2004). Different homodimers or heterodimers of A, B, C and E class proteins interact to form functional 'quartet' protein complexes that are responsible for establishing the various floral organ identities (Pelaz *et al.*, 2000; Honma and Goto, 2001; Theissen, 2001). A subset of plant MADS proteins contains two conserved domains, the MADS domain and the K domain (MIKC type). The latter domain is made up of three amphipathic α-helices, referred to as K1, K2 and K3. Further study showed that the first K-region helix is critical to form *AP3/PISTILLATA* (*PI*)

* For correspondence. E-mail dzl@mail.kib.ac.cn

heterodimers in eudicots (Kramer *et al.*, 1998; Honma and Goto, 2001; Yang *et al.*, 2003; Stellari *et al.*, 2004). Between the MADS and the K domain is an intervening region (I domain). The C-terminal of the MADS-box, the C domain, which shows much lower sequence conservation, plays a key role in forming a higher-order protein complex between dimmers (Kramer *et al.*, 1998; Egea *et al.*, 1999; Honma and Goto, 2001).

The evolution of floral form has been studied quite extensively in both eudicots and monocots (Park *et al.*, 2003, 2004; Irish, 2003; Jack, 2004; Kramer and Hall, 2005). However, many important questions concerning the origin and diversification of flowers have remained largely unanswered (Crane *et al.*, 1995). The construction of phylogenies is a useful approach to study the relationships between development, genes and evolution (Doyle, 1994) and, accordingly, attention has been paid to the evolution and development of floral form in basal angiosperms (Kramer and Irish, 1999, 2000; Kramer *et al.*, 2003; Stellari *et al.*, 2004; Albert *et al.*, 2005; Kim *et al.*, 2005; Li *et al.*, 2005; Zahn *et al.*, 2005).

As a member of Piperales, *Asarum caudigerum* is an important paleoherb. In particular, the species has only one whorl of perianths and its floral form is very similar to the basal-most angiosperm, *Amborella* (Kramer and Irish, 1999, 2000; Stellari *et al.*, 2004). Therefore, it provides an unprecedented opportunity to study the origin and evolution of angiosperm flowers. In this study, the aim was to isolate floral related genes from *A. caudigerum*, to infer evolutionary relationships among florally expression-related genes, and to illustrate the origin and diversification of flowers in angiosperms.

MATERIALS AND METHODS

Plant materials, total RNA extraction and RNA isolation

Asarum caudigerum was grown in the Botanical Garden of the Kunming Institute of Botany (Kunming City, China). Leaves at seedling stage and floral buds from an inbreeding population were collected and frozen in liquid nitrogen, and then transferred to a refrigerator in the laboratory. Total RNA was isolated from frozen leaves and floral buds using TRIZOL reagent (Shanghai Huashun Company) according to the manufacturer's protocol. The ratio of 28S : 18S RNA was about 2 : 1 to prepare total RNA. Poly(A) RNA was purified from the isolated total RNA using an Oligotex mRNA Purification Kit (Qiagen).

Construction of the cDNA library

A PCR-select cDNA Subtraction Kit (Clontech BD) was used to generate a subtractive floral cDNA library. In the forward subtraction of the suppression subtractive hybridization, 2 mg of mRNA from the floral bud was used as a tester and that from leaves as a driver. In the reverse subtraction, the tester and driver were interchanged. cDNA subtraction was performed according to the manual from Clontech. In order to evaluate the efficiency of cDNA subtraction, the conserved gene 26S rRNA of *A. caudigerum* was amplified in the subtracted and un-subtracted cDNA populations, respectively. The 26S rRNA sense primer was 5′-ACTGTCCCTGTCTACTATCC-3′ and the anti-sense primer was 5′-TCAACAAGAACACCACCA-3′. The secondary PCR products from the forward subtraction were directly inserted into pGEM_T-easy Vector (Promega). The cDNA inserts were amplified by polymerase chain reaction (PCR; Perkin-Elmer GeneAmp PCR System 9600) using the nested PCR primer 1 and 2R provided in the PCR-selected cDNA subtraction kit, which were complementary to sequences flanking both sides of the cDNA insert. Thermo-cycling conditions were as follows: an initial denaturation at 94 °C for 3 min, followed by 28 cycles of 95 °C for 10 s, 68 °C for 3 min, and post-heating at 72 °C for 7 min.

DNA sequencing and data analysis

A total of 1920 randomly collected clones were sequenced by the Hua Da Genomic Company (Beijing, China) and a cDNA library was constructed for *Asarum caudigerum*. These sequences were aligned by using Clustal X (Thompson *et al.*, 1997). Nucleotide sequences with a similarity over 90 % were regarded as polymorphisms or repeated sequences and were then removed, out of which relatively long sequences were selected and assembled using the DNAStar program (DNAStar Inc.). To determine the function of these ESTs, nucleic acid and protein homology searches were performed using the BLASTx programs against the NCBI database (http://www.ncbi.nlm.nih.gov/database, as of 1–10 May, 2005). In this study, *e*-values less than 1*e*-5 with more than 100 nucleotides in the ESTs were considered significant. All homologous sequences were collected for further comparisons and analyses.

Data deposition and phylogenetic analysis

Unique ESTs reported in this paper are deposited in the NCBI EST database of GenBank with accession numbers of DV038159-DV038720 and DV075851-DV075856 (http://www.ncbi.nlm.nih.gov/dbEST). A total of 38 putative MADS-box transcription factors isolated from *A. caudigerum* in this study were aligned with those closely related to B-class genes and MADS-box genes from GenBank by using Clustal X (Thompson *et al.*, 1997) and the Alignment Exporter of MEGA version 3.0 (Kumar *et al.*, 2004). Maximum-parsimony (MP) trees were constructed with the pairwise deletion option and close-neighbour interchange (CNI; level = 1) with the initial tree by Random addition (ten replicates) by using MEGA3 (Kumar *et al.*, 2004). Genetic distances were estimated under the Tajima–Nei model. The consensus trees were constructed from 250 bootstrap replicates for the MP.

RESULTS

Suppression subtractive hybridization (SSH) and analysis of subtraction efficiency

Both of the first and second subtracted and unsubtracted PCR products were successfully subtracted (Fig. 1). In

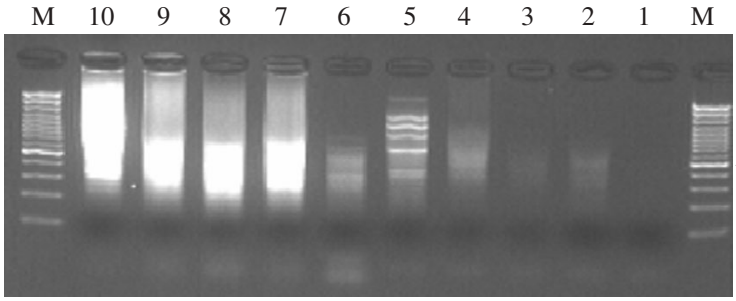

FIG. 1. The first and second of subtracted and unsubtracted PCR product. M, marker size (GeneRuler^{TM} 100 bp DNA ladder); 1, the first PCR products of forward subtraction; 2, the first PCR products of reverse subtraction; 3, the first PCR products of forward unsubtraction; 4, the first PCR products of reverse unsubtraction; 5, the first PCR products of the control provided by the kit; 6, the second PCR products of forward subtraction; 7, the second PCR products of reverse subtraction; 8, the second PCR products of forward unsubtraction; 9, the second PCR products of reverse unsubtraction; 10, the second PCR products of the control provided by the kit.

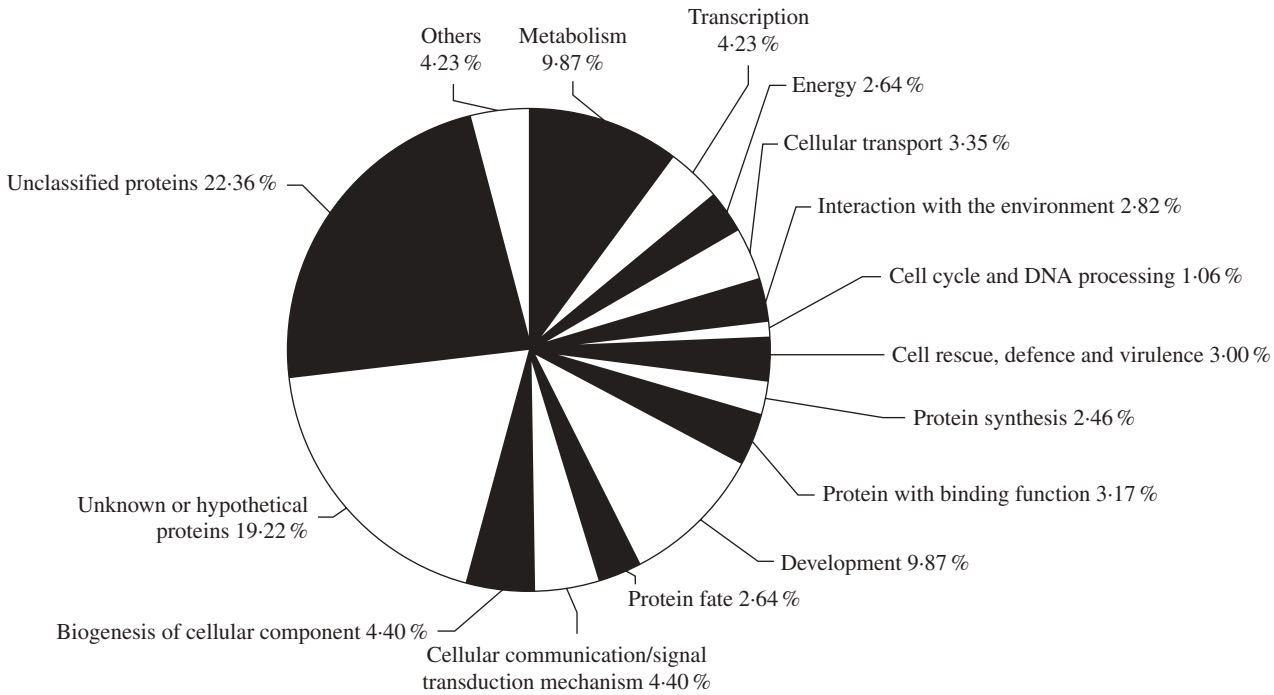

FIG. 2. Functional classification of the unique ESTs.

addition, an analysis indicated that the conserved gene 26S rRNA from *A. caudigerum* was subtracted efficiently. For the unsubtracted cDNA library, the conserved gene 26S rRNA products could be detected after 18 cycles of amplification. However, in subtracted samples, the corresponding bands appeared ten cycles later.

Sequencing and blast results

A total of 1920 clones were sequenced, and 1426 sequences were obtained that were longer than 100 bp. These sequences were further compared with those in nucleotide and protein databases by performing BLASTx searches. Homologies that showed *e*-values less than 1*e*-5 with more than 100 nucleotides in the ESTs were considered significant. Eventually 567 unique ESTs were found. According to functional categories of the *Arabidopsis* proteins (http://mips.gsf.de/proj/thal/db/index.html), cDNA with protein homologies were classified into 16 groups (Fig. 2). Of these groups, 127 genes were putatively novel genes (22.39 %). In addition, 109 genes were annotated as hypothetical or unknown proteins (19.22 %), 56 as development and metabolism proteins (9.87 %), 25 each were annotated as cellular component and cellular communication/signal transduction mechanism proteins (4.40 % in each case) and 24 as transcription proteins (4.23 %) and other proteins.

Phylogenetic reconstruction

The results indicate that 29 genes contained the diagnostic sequences characteristic of the *AP3* subfamily, and the nine other genes formed two clusters with low bootstrap support. Accordingly, two phylogenetic trees were

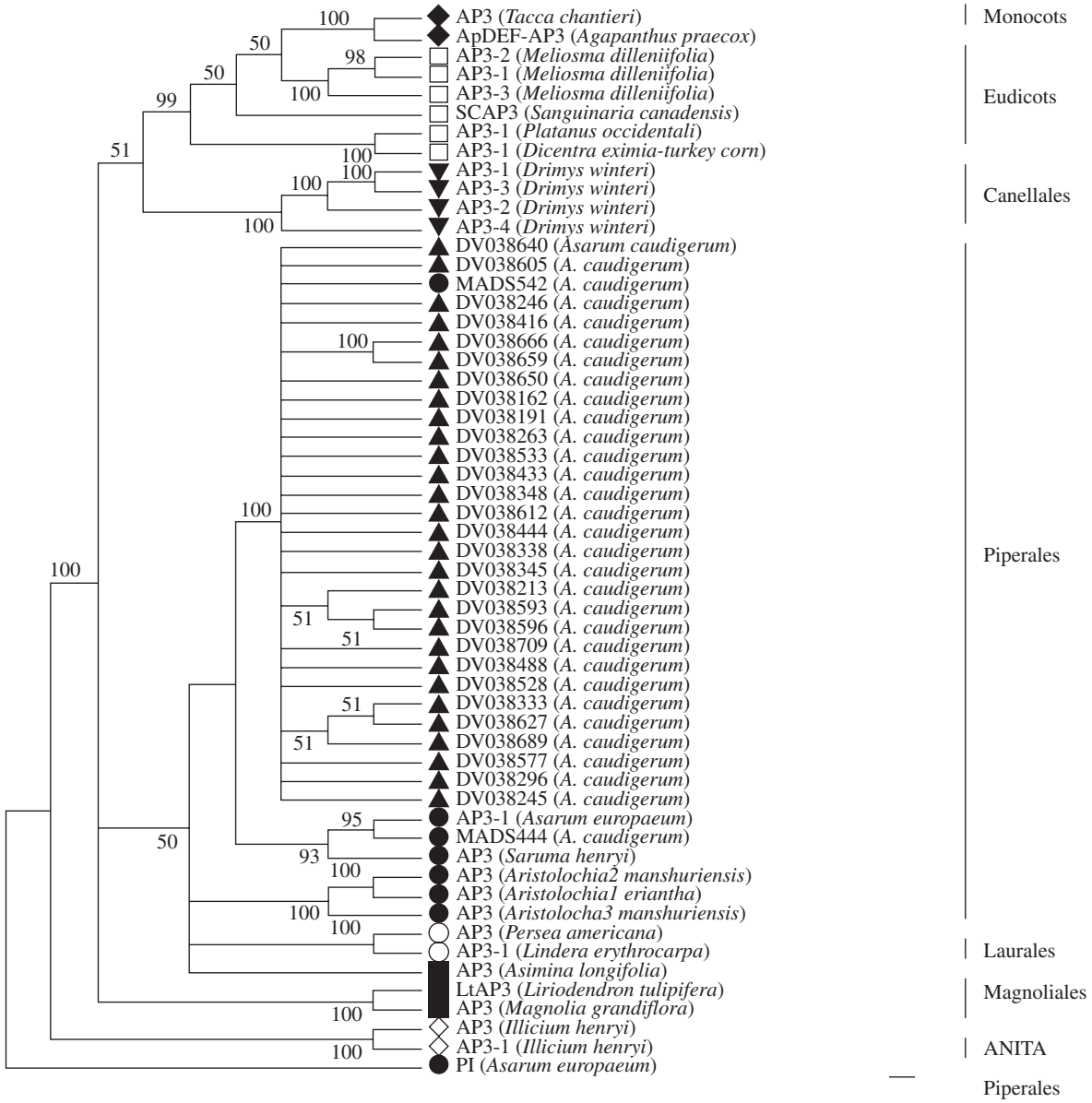

FIG. 3. Consensus tree of the 56 most-parsimonious trees for *A. caudigerum* based on nucleotide sequences of *AP3*. The phylogenetic tree was constructed using the Tajima–Nei model. Only bootstrap percentages at 50 % or higher are shown. CI = 0.472561, RI = 0.676030, RCI = 0.319465. A black triangle represents genes that were isolated from *A. caudigerum* in this study.

generated. Maximum-parsimony close-neighbour-interchange (CNI) with a search of the *AP3* subfamily nucleic acid alignment yielded high bootstrap support between 29 putative MADS-box transcription factors isolated from *A. caudigerum* and the *AP3* subfamily genes, especially *AP3-1* from *A. europaeum*. However, there was no bootstrap support with *PI* from *A. europaeum* (Fig. 3). The bootstrap consensus tree of MADS-box transcription factors indicates that nine putative MADS-box transcription factors isolated from *A. caudigerum* and the others formed two clusters. The MADS-box transcription factors from *A. caudigerum*, *Amborella* and gymnosperms were grouped as one cluster with moderate bootstrap support between *A. caudigerum* and *Amborella* (78 %; Fig. 4).

DISCUSSION

Construction of the cDNA library

The expressed sequence tags (ESTs) reported are the first from the transcriptome of *A. caudigerum*, a species with an interesting phylogenetic position between the angiosperms and gymnosperms. It is noticeable that a considerable number of the genes identified are uncharacterized novel genes, or at least hypothetical proteins with unknown gene function (Fig. 2). Among these 567 unique genes from the subtracted floral cDNA library, up to 22.36 % failed to show significant similarity to any published sequences in GenBank, whereas 19.22 % were annotated as hypothetical or unknown proteins. 28.04 % have predicted functions, and only 31.21 % genes encode proteins with known functions.

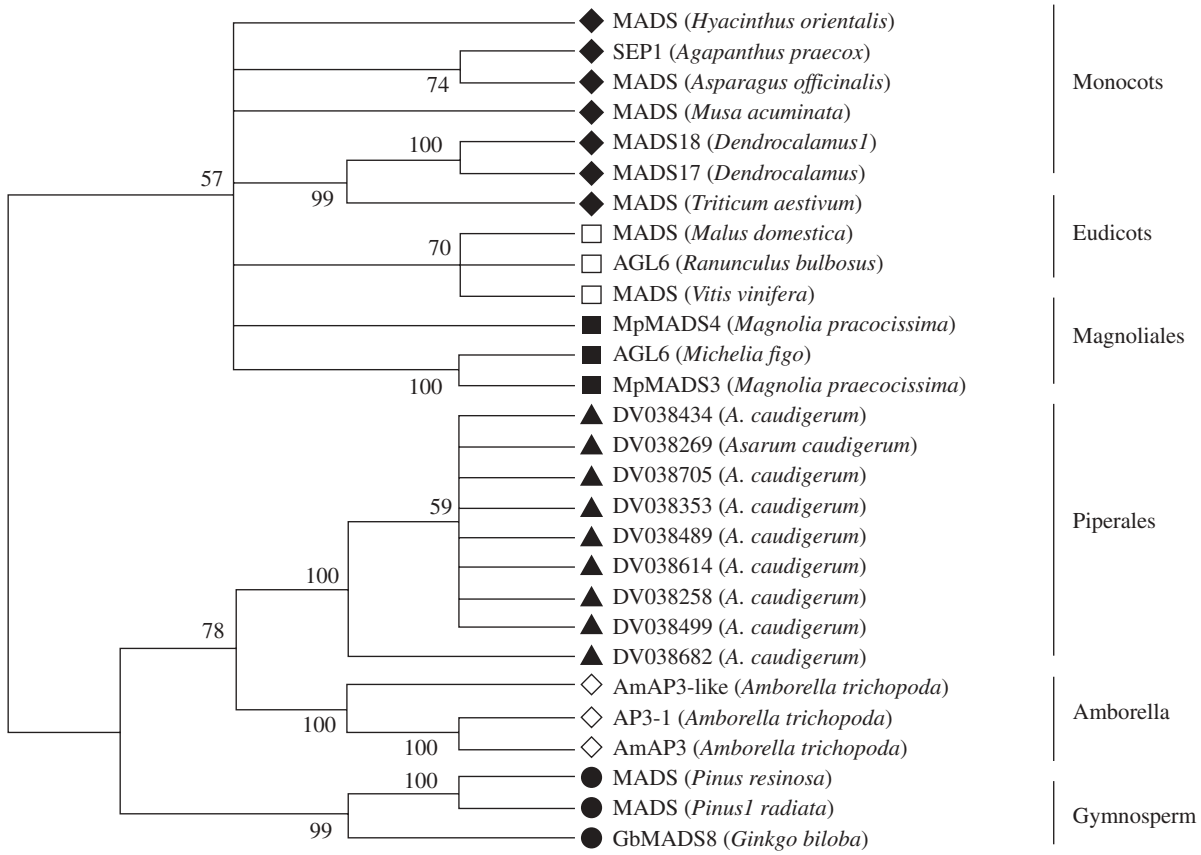

FIG. 4. Consensus tree of the 28 maximum-parsimony close-neighbour-interchanges (CNI) using a search of *A. caudigerum* based on nucleotide sequences of MADS-box transcription factors. The phylogenetic tree was constructed using the Tajima–Nei model. Only bootstrap percentages at 50 % or higher are shown. CI = 0.630216, RI = 0.853561, RCI = 0.537928. A black triangle represents genes that were isolated from *A. caudigerum* in this study.

The results from suppression subtractive hybridization (SSH) and further analyses apparently indicate the high success and efficiency of subtraction for *A. caudigerum* in this study. In the unsubtracted cDNA library, for example, PCR products could be detected after 18 cycles of amplification. In the subtracted cDNA library, however, the corresponding bands appeared after 28 cycles of amplification for the conserved gene 26S rRNA of *A. caudigerum*. This indicates that the high-abundance expressed genes were dramatically reduced while low-abundance ones were rapidly increased. Because the majority of florally expression-related genes are low-abundance-expressed, SSH has been proven a useful method to isolate the florally expression-related genes (Hu *et al.*, 2003). However, it should be pointed out that, like other PCR-based methods, SSH may produce false positives when it is applied to isolating subtracted expressed genes. Therefore, a combination of SSH and cDNA microarray can be a complementary way to identify subtracted expressed genes (Yang *et al.*, 1999).

The evolution of the MADS-box transcription genes

Basal angiosperms are very important for studying the origin, diversification and evolution of angiosperms. It has long been demonstrated that MADS-box transcription factors are critical in the network of flower development (Theissen *et al.*, 2000; Theissen, 2001; Irish, 2003; Litt and Irish, 2003; Nam *et al.*, 2003). So far, about 107 MADS-box transcription genes have been characterized in *Arabidopsis thaliana* and 71 in *Oryza sativa* (Riechmann *et al.*, 2000; Albert *et al.*, 2005; Goff *et al.*, 2002). In the cDNA library we constructed, a total of 38 putative MADS-box transcription factors were isolated from *A. caudigerum*. Of them, 29 showed strong bootstrap support with *AP3-1* from *A. europaeum*. As a member of Piperales, phylogenetic analysis clearly suggested that this species is located at a basal position of angiosperms (Angiosperm Phylogeny Group, 2003; Stellari *et al.*, 2004). Our results indicate that the 29 putative MADS-box transcription factors isolated from *A. caudigerum* were members of the *AP3* subfamily. *AP3* is the B-class of homeotic genes that control petaloid organ identity. B-class homeotic genes are conserved in the high eudicots, but they may not be conserved in the basal angiosperms (Kramer and Irish, 2000; Kramer *et al.*, 2003). In the higher eudicots, the B-class genes, which are represented by homologues of the *A. thaliana AP3* and *PI* genes—the products of *AP3* and *PI* homologues—form heterodimers to control petal and stamen identification in the second and third whorls (Kramer and Irish, 2000). In the eudicots, the first α-helices of the K-domain are key in forming *AP3*/*PI* heterodimers, whose positions 118 and 113 are *AP3* and *PI*

homologs, respectively (Kramer *et al.*, 1998; Honma and Goto, 2001; Yang *et al.*, 2003; Stellari *et al.*, 2004). But in the majority of magnoliid and ANITA clades, *AP3* homologs encode amino acids both at positions of 113 and 118 to function as homodimers (Yang *et al.*, 2003). In our study, there was a high bootstrap support between putative MADS-box transcription factors we isolated from *A. caudigerum* and *AP3* from *A. europaeum*, while no bootstrap support was obtained for *PI* from *A. europaeum*. Phylogenetic analysis of *AP3*- and *PI*-related genes indicates that *AP3*/*PI* duplication occurred after the last common ancestor of angiosperms and gymnosperms (Kramer *et al.*, 1998; Stellari *et al.*, 2004).

As a final remark, *A. caudigerum* showed a very similar floral structure to *Amborella*, whose position is at the very base of the angiosperms (Kramer and Irish, 1999, 2000; Angiosperm Phylogeny Group, 2003; Stellari *et al.*, 2004). Hence the genus *Asarum* has long been used for performing phylogenetic reconstruction and studying the origin and evolution of angiosperm flowers. Using parsimony analysis, our results indicate that the MADS-box transcription factors can be grouped into two major clusters. One cluster included members of MADS-box transcription subfamily from *A. caudigerum*, *Amborella* and the gymnosperms, with moderate bootstrap support between *A. caudigerum* and *Amborella*. It suggests that the origin of *A. caudigerum* may be in the split between the angiosperms and gymnosperms. Another cluster included the members of MADS-box transcription subfamily that are homologous to those from Magnoliales, educots and monocots. Increasing evidence suggests that functional genes that are responsible for flower development may be homologous to each other (Yang *et al.*, 2003; Stellari *et al.*, 2004). Hence our on-going efforts are aimed at characterizing several closely related novel floral genes that may be associated with possible function in the flower development of *A. caudigerum* in order to gain a deeper insight into the origin and evolution of angiosperm flowers.

ACKNOWLEDGEMENTS

We would like to thank Song Ge (Institute of Botany, the Chinese Academy of Sciences) for helpful discussions and comments on the manuscript. This research was supported by the Ministry of Science and Technology of China (award 2004DKA30430).

LITERATURE CITED

Albert VA, Soltis DE, Carlson JE, Farmerie WG, Wall PK, Ilut DC, *et al.* 2005. Floral gene resources from basal angiosperms for comparative genomics research. *BMC Plant Biology* **5**: 1–15.

Angiosperm Phylogeny Group. 2003. An update of angiosperm phylogeny group classification for the orders and families of flowering plants: APG II. *Botanical Journal of the Linnean Society* **141**: 399–436.

Becker A, Theissen G. 2003. The major clades of MADS-box genes and their role in the development and evolution of flowering plants. *Molecular Phylogenetic and Evolution* **29**: 464–489.

Bowman JL, Smthy DR, Meyerowitzetz EM. 1991. Genetic interactions among floral homeotic genes of *Arabidopsis*. *Development* **112**: 1–20.

Coen ES, Meyerowitz EM. 1991. The war of whorls: genetic interactions controlling flower development. *Nature* **353**: 31–37.

Crane PR, Friis EM, Pedersen R. 1995. The origin and early diversification of angiosperms. *Nature* **374**: 27–33.

Ditta G, Pinyopich A, Robles P, Pelaz S, Yanofsky FM. 2004. The SEP4 gene of *Arabidopsis thaliana* functions in floral organ and meristem identity. *Current Biology* **14**: 1935–1940.

Doyle JJ. 1994. Evolution of a plant homeotic multigene family: towards connecting molecular systematics and molecular developmental genetics. *Systematic Biology* **43**: 307–328.

Egea MC, Saedler HE, Sommer H. 1999. Ternary complex formation between the MADS-box proteins SQUAMOSA, DEFICIENS and GLOBOSA is involved in the control of floral architecture in Antirrhinum majus. *EMBO Journal* **18**: 5370–5379.

Ferrario S, Immink GH, Shchennikova A, Jacqueline BL, Angenent GC. 2003. The MADS box gene FBP2 is required for SEPALLATA function in *Petunia*. *Plant Cell* **15**: 914–925.

Goff SA, Ricke D, Lan TH, Presting G, Wang R, Dunn M, *et al.* 2002. A draft sequence of the rice genome (*Oryza sativa L. ssp. japonica*). *Science* **296**: 92–100.

Honma T, Goto KJ. 2001. Complexes of MADS-box proteins are sufficient to convert leaves into floral organ. *Nature* **409**: 525–529.

Hu W, Wang Y, Bowers C, Ma H. 2003. Isolation, sequence analysis, and expression studies of florally expressed cDNA in *Arabidopsis*. *Plant Molecular Biology* **53**: 454–463.

Irish FV. 2003. The evolution of floral homeotic gene function. *BioEssays* **25**: 637–464.

Jack T. 2004. Molecular and genetic mechanisms of floral control. *Plant Cell* **16**: 1–17.

Kim ST, Koh J, Yoo MJ, Kong HZ, Hu Y, Ma H, Soltis PS, Soltis DE. 2005. Expression of floral MADS-box genes in basal angiosperms: implications for the evolution of floral regulators. *Plant Journal* **43**: 724–744.

Kramer EM, Hall JC. 2005. Evolutionary dynamics of genes controlling floral development. *Current Opinion in Plant Biology* **8**: 13–18.

Kramer EM, Irish FV. 1999. Evolution of genetic mechanisms controlling petal development. *Nature* **399**: 144–148.

Kramer EM, Irish FV. 2000. Evolution of the petal and stamen developmental programs: evidence from comparative studies of the lower eudicots and basal angiosperms. *International Journal of Plant Sciences* **161**: 29–40.

Kramer EM, Robert LD, Vivian FI. 1998. Molecular evolution of genes controlling petal and stamen development: duplication and divergence within the *APETALA3* and *PISTILLATA* MADS-box gene lineages. *Genetics* **149**: 765–783.

Kramer EM, Stilio VS Di, Schluter P. 2003. Complex patterns of gene duplication in the *APETALA3 and PISTILLATA* lineages of the Ranunculaceae. *International Journal of Plant Sciences* **164**: 1–11.

Krizek BA, Meyerowitz EM. 1996. The *Arabidopsis* homeotic genes *APETALA3* and *PISTILLATA* are sufficient to provide the B class organ identity function. *Development* **122**: 11–22.

Kumar S, Tamura K, and Nei M. 2004. MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. *Briefings in Bioinformatics* **5**: 150–163.

Li GS, Zahn LM, Kong HZ, Chen AD, Theisssen G, Lu AM. 2005. Characterization of candidate class A, B and E floral homeotic genes from the perianthless basal angiosperm Chloranthus spicatus (Chloranthaceae). *Developmental Evolution* **215**: 437–449.

Litt A, Irish VF. 2003. Duplication and diversification in the *APETALA1/FRUITFULL* floral homeotic gene lineage: implications for the evolution of floral development. *Genetics* **165**: 821–833.

Meyerowitz EM, Smath DR, Bowman JL. 1989. Abnormal flowers and pattern formation in floral development. *Development* **106**: 209–217.

Mizukami Y, Ma H. 1992. Ectopic expression of the floral homeotic gene *AGAMOUS* in transgenic Arabidopsis plants alters floral organ identity. *Cell* **71**: 119–131.

Nam Jm, dePamphilis CW, Ma H, Nei M. 2003. Antiquity and evolution of the MADS-box gene family controlling flower development in plant. *Molecular Biology and Evolution* **20**: 1435–1447.

Park JH, Ishikawa Y, Hida R. 2003. Expression of *AODEF*, a B-functional MADS-box gene, in stamens and inner tepals of the dioceious species *Asparagus officinalis* L. *Plant Molecular. Biology* **51**: 867–875.

Park JH, Ishikawa Y, Ochiai T, Kanno A, Kameya T. 2004. Two *GLOBOSA*-like genes are expressed in second and third whorls of

homo chlamydeous flowers in *Asparagus officinalis* L. *Plant and Cell Physiology* **45**: 325–332.

Pelaz S, Ditta G, Baumann E, Wisman E, Martin FY. 2000. B and C floral organ identity functions require *SEPALLATA* MADS-box genes. *Nature* **405**: 200–202.

Pelaz S, Rosalinda TL, Elena RA, Martin FY. 2001. Conversion of leaves into petals in *Arabidopsis*. *Current Biology* **11**: 182–184.

Riechmann JL, Heard J, Martin G, Jan TK, Thomas M, Winter KU, Heinz S. 2000. *Arabidopsis* transcription factors: genome-wide comparative analysis among eukayotes. *Science* **290**: 2105–1110.

Stellari MG, Jaramillo MA, Kramer ME. 2004. Evolution of the APETALA3 and PISTILLATA lineages of MADS-box-containing genes in the basal angiosperms. *Molecular Biology and Evolution* **21**: 506–519.

Theissen G. 2001. Development of floral organ identity: stories from the MADS house. *Current Opinion in Plant Biology* **4**: 75–85.

Theissen G, Becker A, Rosa AD, Kanno A, Kim JT, Munster T, Winter KW, Saedler H. 2000. A short history of MADS-box genes in plants. *Plant Molecular Biology* **42**: 115–149.

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG. 1997. The CLUSTAL X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research* **25**: 4876–4882.

Thorne R. 1992. Classification and geography of the flowering plants. *Botanic Review* **58**: 225–348.

Weigel D. 1998. From floral induction to floral shape. *Current Opinion in Plant Biology* **1**: 55–59.

Yang GP, Ross DT, Kang WW Brown PO, Weigel RJ. 1999. Combinating SSH and cDNA microarrays for rapid identification of differentially expressed genes. *Nucleic Acids Research* **27**: 1517–1523.

Yang YZ, Fanning L, Jack T. 2003. The K domain mediates heterodimerization of the Arabidopsis floral organ identity proteins, APETALA3 and PISTILLATA. *Plant Journal* **33**: 47–59.

Zahn LM, Kong H, Leebens-Mack JH, Kim S, Soltis PS, Landherr LL, Soltis DE, dePamphilis CW, Ma H. 2005. The evolution of the *SEPALLATA* subfamily of MADS-box genes: a pre-angiosperm origin with multiple duplications throughout angiosperm history. *Genetics* **169**: 2209–2223.